IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	: Hsing-Pang Hsieh	Art Unit	: 1617
Serial No.	: 10/817,490	Examiner	: Yong Soo Chong
Filed	: April 2, 2004	Conf. No.	: 2330
Title	: Treatment Of Hepatitis C Virus Infection With Sesquiterpene Lactones		

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF SUI-YUAN CHANG UNDER 37 C.F.R. 1.132

I, Sui-Yuan Chang, declare:

1. I hold the positions of Associate Professor at the Department of Clinical Laboratory Sciences and Medical Biotechnology, National Taiwan University, and Supervisor at the Department of Laboratory Medicine, National Taiwan University Hospital. Prior to these positions, I earned a doctoral degree from the Department of Immunology and Infectious Diseases, Harvard School of Public Health.

2. My current research focuses on genotypic analysis of viruses and therapy of virus infections. Overall, I have studied virus infections for 10 years. .

3. I have reviewed U.S. Serial No. 10/817,490. This application includes claims covering a method of treating hepatitis C virus (HCV) infection with an effective amount of a sesquiterpene lactone compound.

4. I have also reviewed Hwang et al., US Patent 5,905,089 (Hwang) and Baba et al., US Patent 6,123,943 (Baba), both of which are cited in U.S. Serial No. 10/817,490.

Hwang discloses using sesquiterpene lactone compounds to inhibit NF-κB activity. Baba suggests using 1,2,3,4-tetrahydroisoquinoline compounds to treat a large number of diseases, including viral hepatitis and cytomegalovirus hepatitis, via inhibiting

NF-kB activity. Neither Hwang nor Baba teaches using any sesquiterpene lactone compound to treat HCV infection.

5. In view of the teachings of Hwang and Baba and based on my scientific knowledge, I conclude that a skilled person in the art would not have expected that sesquiterpene lactone compounds taught in Hwang, which are different from 1,2,3,4-tetrahydroisoquinonline compounds taught in Baba, can be used in treating the various diseases mentioned in Baba, e.g., viral hepatitis and cytomegalovirus hepatitis, let alone HCV infection (which is not mentioned in that reference).

6. I and my associates have assessed the activity of a sesquiterpene lactone compound, i.e., parthenolide, against cytomegalovirus (CMV), which is specifically mentioned in Baba, by conducting two assays.

The procedure of the first assay follows: Vero cells were first incubated with CMV at MOI of 0.2 in MEM at 37°C for 1 h. They were then incubated in the presence or absence of 2 μM parthenolide at 37°C for 1 h. The cells were washed thoroughly to remove the non-absorbed viruses and further cultured in a fresh medium. The supernatant was collected from the cell culture at the 6th day post infection (dpi). Viral DNA in the supernatant was extracted and amplified by PCR using a CMV UL97 primer pair.

The first assay shows that parthenolide did not significantly repress CMV production at the 6th dpi. See Figure 1 (attached).

The procedure of the second assay follows: CMV-infected MRC5 cells were cultured in the presence or absence of parthenolide in the same manner as described above. Cytopathic effects (CPEs) were observed at different time points during the culture.

The second assay shows that parthenolide did not prevent the development of CPE. More specifically, the cell culture exhibited a mild CPE at the 3rd dpi, an obvious

CPE at the 4th dpi, and a severe CPE at the 6th dpi, whether parthenolide was present or not. See Figures 2-4 (also attached).

The results of the above two assays compel the conclusion that parthenolide, a sesquiterpene lactone compound taught in Hwang, cannot be used to treat CMV hepatitis disclosed in Baba.

7. All statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Respectfully submitted,

Date: October 19, 2009

Sui-Yuan Chang, D.Sc.
Associate Professor
Department of Clinical Laboratory
Sciences and Medical Biotechnology
National Taiwan University
Supervisor
Department of Laboratory Medicine,
National Taiwan University Hospital

Customer No. 69713
Occhiuti Rohlicek & Tsao LLP
10 Fawcett Street
Cambridge, MA 02138
Telephone: (617) 500-2500
Facsimile: (617) 500-2499

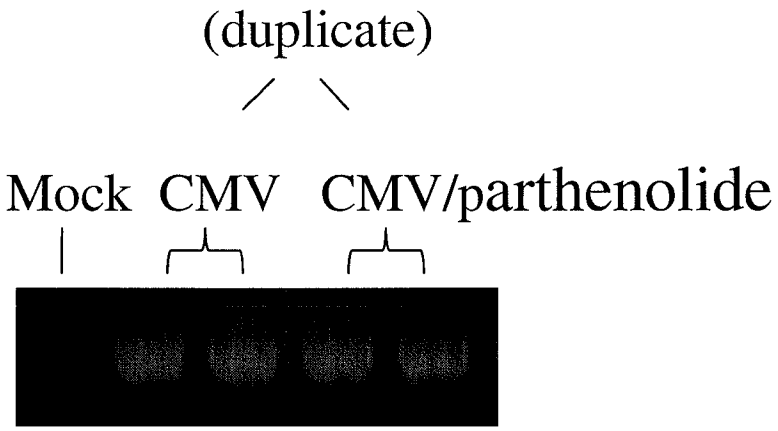


Figure 1

CMV-infected MRC5 cells (3 dpi)

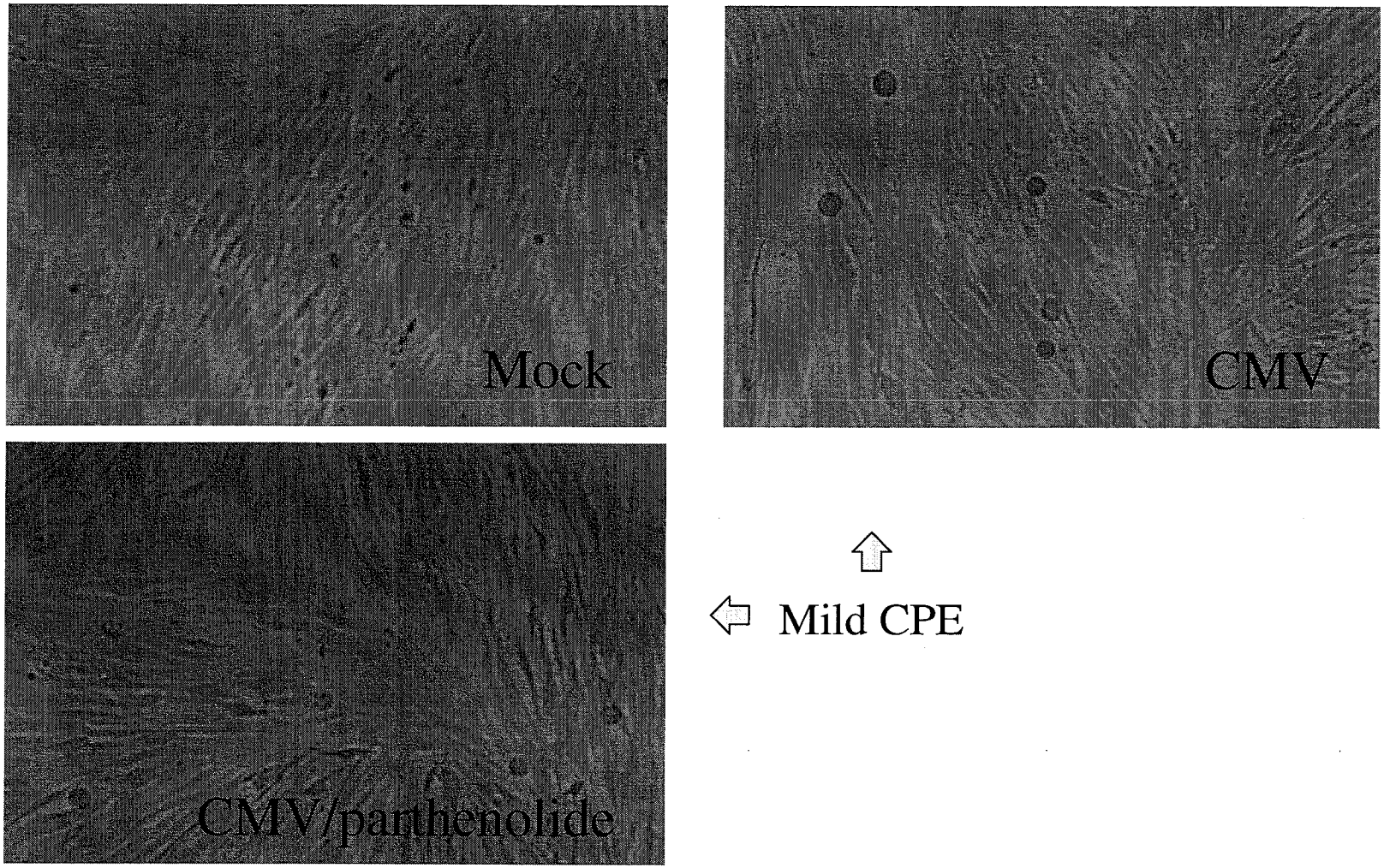
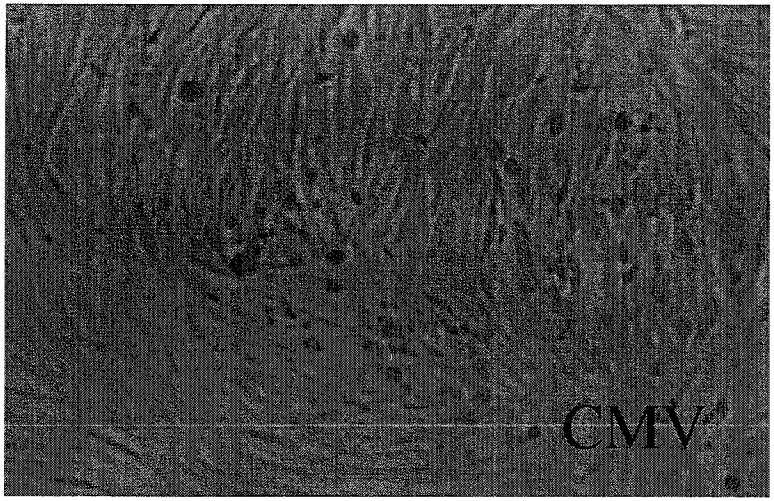
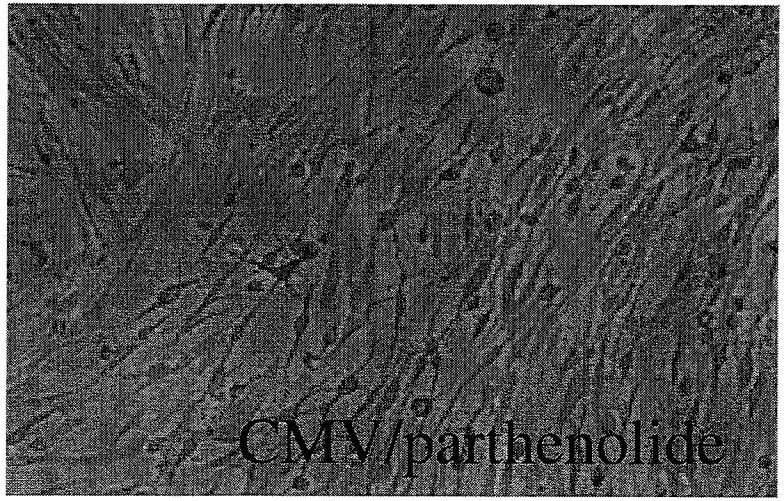


Figure 2

CMV-infected MRC5 cells (4 dpi)





⇧

⇦ Obvious CPE

Figure 3

CMV-infected MRC5 cells (6 dpi)

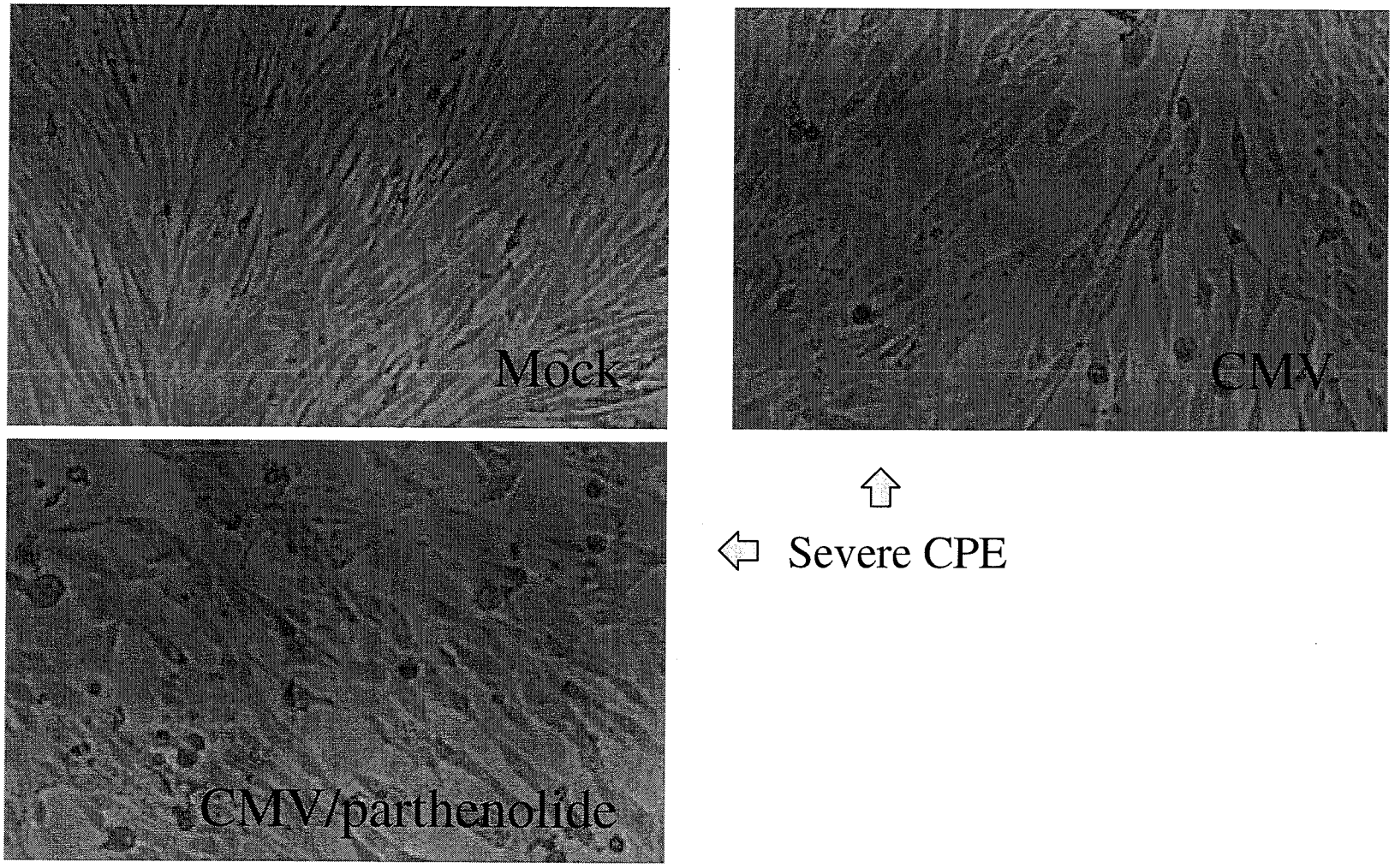


Figure 4

Journal of Neurochemistry
Lippincott-Raven Publishers, Philadelphia

Aspirin and Salicylate Protect Against MPTP-Induced Dopamine Depletion in Mice

Nadine Aubin, Olivier Curet, Annie Deffois, and Chris Carter

Central Nervous System Research Department, Synthélabo Recherche, Bagneux, France

Abstract: The neurotoxic effects of the dopamine-selective neurotoxin MPTP (15 mg/kg, s.c.), in mice, were totally prevented by systemic administration of salicylate (ED_{50} = 40 mg/kg, i.p.), aspirin (ED_{50} = 60 mg/kg, i.p.), or the soluble lysine salt of aspirin, Aspegic (ED_{50} = 80 mg/kg, i.p.). The protective effects of aspirin are unlikely to be related to cyclooxygenase inhibition as paracetamol (100 mg/kg, i.p.), diclofenac (100 mg/kg, i.p.), ibuprofen (20 mg/kg, i.p.) and indomethacin (100 mg/kg, i.p.) were ineffective. Dexamethasone (3–30 mg/kg, i.p.), which, like aspirin and salicylate, has been reported to inhibit the transcription factor NF-$\kappa\beta$, was also ineffective. Aspirin or salicylate (100 μM) had no effect on dopamine uptake into striatal synaptosomes or on monoamine oxidase B activity. The neuroprotective effects of salicylate derivatives could perhaps be related to hydroxyl radical scavenging. This was suggested by the fact that hydroxylated metabolites of salicylate (2,3- and 2,5-dihydrobenzoic acid) were recovered in brain tissue following the combined administration of MPTP and aspirin to a greater extent than following aspirin alone. The surprising neuroprotective effects of aspirin in an animal model of Parkinson's disease warrant further clinical investigation. **Key Words:** Aspirin—Salicylic acid—1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine—Mouse—Neurotoxicity—Parkinson's disease model.
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Since the chance discovery of the neurotoxic effects of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in humans (Langston and Ballard, 1983), it has been thought that a similar type of environmental toxin might be responsible for idiopathic Parkinson's disease and reasoned that an understanding of the neurotoxic effects of MPTP might lead to prevention of this debilitating disorder. MPTP is metabolized to the 1-methyl-4-phenylpyridinium ion (MPP^+) by monoamine oxidase (MAO) B (Langston et al., 1984), and this highly toxic metabolite is selectively taken up into dopaminergic neurons via the dopamine (DA) transporter (Snyder and D'Amato, 1986). MPP^+ is a mitochondrial toxin that selectively inhibits complex 1 of the respiratory chain (Cleeter et al., 1992), leading evidently to energy compromise and to the production of potentially cytotoxic free radicals (Jenner, 1991; Adams et al., 1993; Bowling and Beal, 1995). Accumulating evidence suggests that oxidative stress is also a feature of Parkinson's disease neuropathology (Dexter et al., 1986; Jenner, 1991; Fahn and Cohen, 1992; Schapira, 1994; Bowling and Beal, 1995), and mitochondrial complex 1 deficiency is also a feature of this disease (Mizuno et al., 1989; Schapira et al., 1990). Numerous studies in rodents or primates (including, of course, the original observation in humans) have shown that MPTP produces a selective lesion of the nigrostriatal DA system that closely mimics the neuropathological and symptomatic sequelae of Parkinson's disease. Blockade either of MAO-B (Rose et al., 1989) or of DA uptake (Bradbury et al., 1985) is known to protect against the neurotoxic effects of MPTP in laboratory animals, although recent clinical trials with the selective MAO-B inhibitor deprenyl have met with mixed success (Olanow et al., 1995; Parkinson Study Group, 1996). This type of clinical approach does not attack the neurotoxic process itself [although antiapoptotic and free radical scavenging effects have recently been attributed to deprenyl (Tatton et al., 1994; Gerlach et al., 1996)] but aims rather to prevent the metabolism of a putative (and unknown) MPTP-like molecule to a toxic metabolite.

Several arguments suggest that the neurotoxic effects of MPTP (or MPP^+) might be mediated via free radical production. This may result from disturbances

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Address correspondence and reprint requests to Dr. C. Carter at Central Nervous System Research Department, Synthélabo Recherche, 31 Avenue PV Couturier, B.P. 110, 92225 Bagneux Cedex, France.

Abbreviations used: DA, dopamine; DHBA, dihydroxybenzoic acid; DOPAC, 3,4-dihydroxyphenylacetic acid; 5-HIAA, 5-hydroxyindoleacetic acid; 5-HT, 5-hydroxytryptamine (serotonin); HVA, homovanillic acid; MAO, monoamine oxidase; MPP^+, 1-methyl-4-phenylpyridinium ion; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; 3-MT, 3-methoxytyramine; PEA, phenylethylamine.

in oxygen metabolism produced by complex 1 inhibition or from diverse effects of MPTP and MPP^+ on free radical-generating processes (for reviews, see Gerlach et al., 1991; Tipton and Singer, 1993; Zang and Misra, 1993). MPTP decreases glutathione levels and increases the levels of reactive oxygen species and the degree of lipid peroxidation in mouse brain slices in vitro and increases the levels of reactive oxygen species in mouse brain in vivo. MPTP neurotoxicity in vitro is reduced by glutathione (Sriram et al., 1997). In vitro studies have shown that MPP^+ neurotoxicity can be reduced by vitamin E, vitamin C, coenzyme Q, and mannitol (but not by superoxide dismutase, catalase, allopurinol, or dimethyl sulfoxide) (Akaneya et al., 1995). β-Carotene, vitamin C, and *N*-acetylcysteine partially protect against the neurotoxic effects of MPTP in mice (Perry et al., 1985), as do nicotinamide, coenzyme Q, and the free radical spin trap *N-tert*-butyl-α-(sulfophenyl)nitrone (Schulz et al., 1995*a*).

Chuieh and others have demonstrated that MPTP results in the formation of the highly toxic hydroxyl radical in vivo. Hydroxyl radical formation was monitored in dialysis studies by the salicylate detection method (Chiueh et al., 1992, 1993; Obata and Chiueh, 1992). Hydroxyl radicals react with salicylate to form 2,3- and 2,5-dihydroxybenzoic acid (DHBA). MPTP increases the formation of both 2,3- and 2,5-DHBA in vivo. If hydroxyl radicals are interacting with salicylate, then of course salicylate is also mopping up hydroxyl radicals. If indeed these are the agents through which MPTP exerts its neurotoxicity, we reasoned that salicylate should provide some degree of neuroprotection in the MPTP model. We found that salicylate totally protects against the neurotoxic effects of MPTP and that this property is shared by acetylsalicylate (aspirin) and a lysine salt formulation of aspirin, Aspegic, but not by other cyclooxygenase inhibitors.

MATERIALS AND METHODS

Animals and tissues

The MPTP studies were conducted on male C57Bl/6 mice (weighing 20–25 g; Iffa Credo, France). In vitro studies on DA uptake or MAO-B activity were conducted using brain tissue from male Sprague–Dawley rats (weighing 180–250 g; Iffa Credo, France). Animals were housed in a controlled environment (light/dark cycles of 12 h with lights on from 7 a.m. to 7 p.m., temperature of 21 ± 2°C) with food and water ad libitum.

Assay of striatal DA, serotonin [5-hydroxytryptamine (5-HT)], and their related metabolites

Mice were killed by decapitation. Striata were dissected out, frozen, weighed, and stored at −80°C until analysis. 5-HT, DA, homovanillic acid (HVA), 3,4-dihydroxyphenylacetic acid (DOPAC), 3-methoxytyramine (3-MT), and 5-hydroxyindoleacetic acid (5-HIAA) were quantified by HPLC with electrochemical detection. Frozen tissues were sonicated in 800 μl of 0.05 *M* $HClO_4$ containing 0.5 m*M* EDTA, 2 m*M* sodium metabisulfite, and 3,4-DHBA (final concentration, 1 ng/50 μl) as the internal standard. After centrifugation, 50 μl of the supernatant was injected onto the liquid chromatographic column using a refrigerated (4°C) autoinjector (Wisp 712; Waters, Milford, MA, U.S.A.). Separation was achieved at room temperature. The HPLC system consisted of a pump and a stainless steel separation column (0.46 × 7 cm) packed with Ultrasphere XL ODS C18 (particle size, 3 μm; Beckman, Fullerton, CA, U.S.A.). The mobile phase contained 0.1 *M* NaH_2PO_4, 1 m*M* EDTA, 2.5 m*M* octanesulfonic acid, and 7% CH_3CN, pH 3.4. The flow rate was 0.9 ml/min. Electrochemical detection was carried out by means of an amperometric detector (model 460; Waters) with a glassy carbon working electrode and an Ag/AgCl reference electrode. The detector potential was set at 0.8 V versus the reference electrode. Concentrations of each compound were calculated using a computing integrator (Maxima; Waters) with reference calibration curves obtained after injection of standards.

Assay of striatal salicylate, 2,3-DHBA, and 2,5-DHBA

Salicylate and its hydroxylated metabolites were quantified using the methods described by Giovanni et al. (1995). Mice were killed by decapitation. Striata were dissected out, frozen, weighed, and stored at −80°C until analysis. Frozen tissues were sonicated in 150 μl of 0.05 *M* $HClO_4$ containing 0.5 m*M* EDTA and 2 m*M* sodium metabisulfite. After centrifugation, 50 μl of the supernatant was injected onto the liquid chromatographic column using a refrigerated (4°C) autoinjector (Wisp 712; Waters). Separation was achieved at room temperature using a stainless steel separation column (0.46 × 7 cm) packed with Ultrasphere XL ODS C18 (particle size, 3 μm; Beckman). The mobile phase contained 0.1 *M* NaH_2PO_4, 1 m*M* EDTA, 2.25 m*M* octanesulfonic acid, and 5% CH_3CN, pH 3.2. The flow rate was 0.9 ml/min. For 2,3- and 2,5-DHBA the detection was carried out by means of an amperometric detector (model 460; Waters). The detector potential was set at 0.8 V versus the reference electrode. Salicylate was detected with an ultraviolet detector set at 300 nm. Concentrations of each compound were calculated using a computing integrator (Maxima; Waters) with reference calibration curves obtained after injection of standards.

Determination of MAO-A and MAO-B activities

Rat brains were homogenized in 20 volumes of 0.1 *M* sodium phosphate buffer (pH 7.4) at 4°C. MAO-A and MAO-B activities were assayed as previously described (Curet et al., 1996) using [^{14}C]5-HT (final concentration, 125 μ*M*) as the specific MAO-A substrate or phenyl[^{14}C]ethylamine ([^{14}C]PEA; final concentration, 8 μ*M*) as the specific MAO-B substrate.

In vitro measurement of [^{3}H]DA uptake in rat brain synaptosomes

Rat striatal synaptosomes were prepared at 4°C by homogenization in 40 volumes of 0.32 *M* sucrose with 10 strokes of a Teflon/glass homogenizer. After centrifugation at 2,000 *g* for 10 min, the supernatant was centrifuged at 10,000 *g* for 20 min, and the pellet was resuspended in 0.32 *M* sucrose (2 ml) and recentrifuged with 0.8 *M* sucrose (8 ml). The resulting pellet was resuspended in 6 ml of buffer (10 m*M* HEPES, 147 m*M* NaCl, 5 m*M* KCl, 2 m*M* $MgCl_2$, 2 m*M* $CaCl_2$, and 10 m*M* glucose, adjusted to pH 7.4). All subsequent operations were at 25°C. Fifty-microliter aliquots

(~50 μg of protein per well) were distributed into 96-well glass fiber filter plates (MAFCNOB; Millipore SA), and drugs were added in a further 50 μl of the above HEPES buffer following a 10-min incubation. [^{3}H]DA (final concentration, 400 n*M*) was added 10 min before filtration on a Multiscreen system, and synaptosomes were washed twice with 200 μl of HEPES buffer. After direct application of a solid scintillator (MeltiLex; Wallac) to the 96-well glass fiber filter plates, radioactivity was counted in a microplate scintillation counter (MicroBeta 1450 Trilux; Wallac).

Body temperature measurement

Mice core temperature was measured at different times after MPTP or salicylate administration using a small rectal probe and Thermistor thermometer with minimal immobilization stress.

Statistics

The statistical significance of the data was evaluated by ANOVA followed by Dunnett's (homogeneous variances) or Kruskal–Wallis (heterogeneous variances) test.

Chemicals and drugs

[^{14}C]5-HT creatinine sulfate (1.8–2.2 GBq/mmol) was supplied by Amersham (Buckinghamshire, U.K.). [^{14}C]PEA hydrochloride (1.8–2.2 Gbq/mmol), [^{3}H]DA (1,365 GBq/mmol), and Biofluor were purchased from New England Nuclear (Boston, MA, U.S.A.). Salicylic acid, aspirin, diclofenac, dexamethasone, ibuprofen, 2,3-DHBA, 2,5-DHBA, 3,4-DHBA, DA, noradrenaline, DOPAC, 5-HIAA, 3-MT, 5-HT creatinine sulfate, PEA hydrochloride, and 2,5-diphenyloxazole were supplied by Sigma (St. Louis, MO, U.S.A.). Toluene, EDTA, and ethyl acetate were purchased from Labosi (Paris, France). MPTP was obtained from Research Biochemicals International (Natick, MA, U.S.A.). The analytical grade buffers $NaH_2PO_4 \cdot 2H_2O$, $Na_2HPO_4 \cdot 2H_2O$, and perchloric acid were purchased from Merck (Darmstadt, Germany). Aspegic, paracetamol, and indomethacin were synthesized in the Chemistry Department at Synthélabo.

Drug treatments

Aspirin, dexamethasone, paracetamol, diclofenac, and indomethacin were administered intraperitoneally as a suspension in 0.5% Methocel gel plus 0.5% Tween 80 (wt/wt) in a volume of 10 ml/kg. Salicylic acid, Aspegic, ibuprofen, MPTP, and 2,3- and 2,5-DHBA were administered in saline in a volume of 10 ml/kg. Doses always refer to the free base and are expressed in mg/kg of body weight.

RESULTS

Effects of MPTP on DA, 5-HT, and their metabolites in striatum

Mice received graded doses of MPTP (10, 20, 30, 40, and 50 mg/kg, s.c.) and were killed 2 days later. As illustrated in Fig. 1A, MPTP decreased the striatal levels of DA, DOPAC, HVA, and 3-MT in a dose-dependent manner without affecting the levels of 5-HT and 5-HIAA. The maximal decrease of DA levels was obtained with a dose of 50 mg/kg, s.c. Higher doses of MPTP induced mortality. A dose of 15 mg/kg MPTP was chosen for further studies. The time course of the effects of MPTP (15 mg/kg, s.c.) on striatal DA, DOPAC, and 3-MT levels is shown in Fig. 1B. As described by others (Pileblad et al., 1985), MPTP produced a rapid and sustained decrease in striatal DOPAC levels that was maximal 2 h after MPTP injection. DA levels more gradually declined over the 48-h period. DA depletion was sustained for at least 2 weeks (see, e.g., Fig. 3). MPTP also produced a transient increase in striatal 3-MT levels within the first hour following injection. No mortality was observed with this dose of MPTP for up to 2 weeks after MPTP injection.

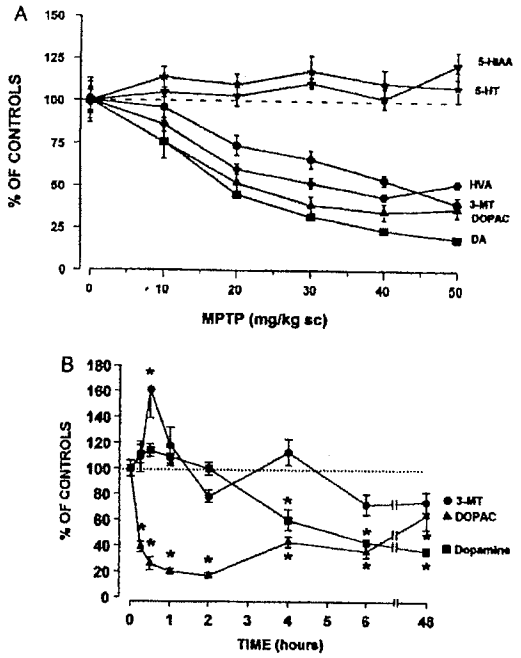

FIG. 1. A: Effects of administration of graded doses of MPTP on striatal levels of DA, DOPAC, HVA, 3-MT, 5-HT, and 5-HIAA in mouse brain. The animals were killed 2 days after administration of MPTP (10–50 mg/kg, s.c.). Data are mean ± SEM (bars) values (n = 8–9), as a percentage of variations versus controls. Control levels (in pg/mg of tissue) were as follows: DA, 13,440 ± 1,147; DOPAC, 1,377 ± 505; 3-MT, 585 ± 109; 5-HT, 442 ± 105; and 5-HIAA, 328 ± 97. **B:** Time course of the effects of MPTP (15 mg/kg, s.c.) on striatal levels of DA, DOPAC, and 3-MT. Animals were killed at defined times following MPTP administration.

Protective effect of aspirin, Aspegic, and salicylate against MPTP-induced DA depletion

In a first experiment, mice were pretreated with different doses of aspirin, Aspegic, and salicylate 1 h before administration of MPTP (15 mg/kg, s.c.). Striatal DA levels were measured 2 days later. As illustrated in Fig. 2, aspirin, Aspegic, and salicylate each prevented MPTP-induced DA depletion in a dose-related manner with ED_{50} values of 60, 80, and 40 mg/kg, i.p., respectively. This protection was total at the highest dose of each drug. The total protective effect of the highest dose of salicylate (100 mg/kg, i.p.) was

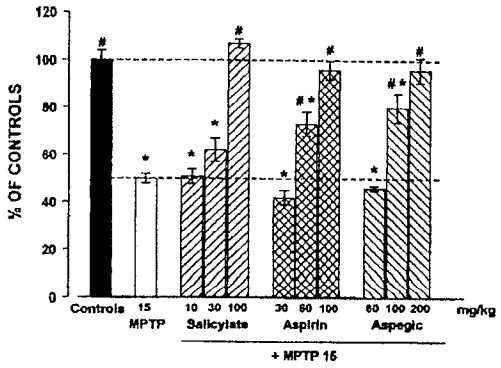

FIG. 2. Effects of systemic intraperitoneal administration of graded doses of salicylate, aspirin, and Aspegic on depletion of DA in striatum of mice induced by MPTP. The animals received a single administration of salicylate (10, 30, or 100 mg/kg, i.p.), aspirin (30, 60, or 100 mg/kg, i.p.), or Aspegic (60, 100, or 200 mg/kg, i.p.) 1 h before MPTP (15 mg/kg, s.c.) and were killed 2 days later. Data are mean ± SEM (bars) values (n = 6). Control DA levels were 14,310 ± 719 pg/mg of tissue. $^{*}p < 0.01$ compared with control; $^{\#}p < 0.01$ compared with MPTP group.

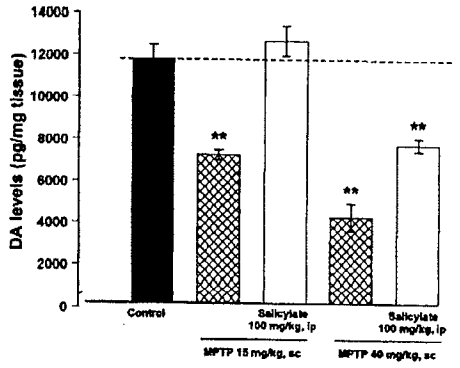

FIG. 4. Effects of salicylate on depletion of DA in striatum of mice induced by different doses of MPTP. The animals received a single administration of salicylate (100 mg/kg, i.p.) 1 h before MPTP (15 or 40 mg/kg, s.c.) and were killed 2 days later. Data are mean ± SEM (bars) values (n = 6). Control DA levels were 11,799 ± 704 pg/mg of tissue. $^{**}p < 0.01$ compared with control.

maintained in mice killed 2 weeks after administration of MPTP (15 mg/kg, s.c.; Fig. 3). Whereas salicylate (100 mg/kg, i.p.) totally blocked the effects of MPTP at 15 mg/kg, s.c., it only partially protected (50%) against the effects of a higher dose (40 mg/kg, s.c.) of the toxin (Fig. 4). To study the time course of the effect of aspirin (60 mg/kg, i.p.), the drug was administered at different times before and after a single dose of MPTP (15 mg/kg, s.c.). As illustrated in Fig. 5, significant protection was observed when aspirin was administered from 2 h before MPTP and for up to 2 h following MPTP administration. The maximal protection was observed when aspirin was given concomitantly or 1 h after MPTP. No protection was seen when aspirin was delayed for >4 h after MPTP administration.

Aspirin (100 mg/kg, i.p.), salicylate (100 mg/kg, i.p.), or Aspegic (200 mg/kg, i.p.) per se had no effect on striatal levels of DA 2 days after dosing (data not shown).

Acute effects of aspirin and MPTP on DA and its metabolites in rat striatum

The neurotoxic effects of MPTP as revealed by chronic DA depletion are not observed until several hours after MPTP administration. However, more immediately after MPTP injection, effects on striatal DA turnover can be observed that reflect the toxin's more acute effects on DA uptake and metabolism, e.g., Fig.

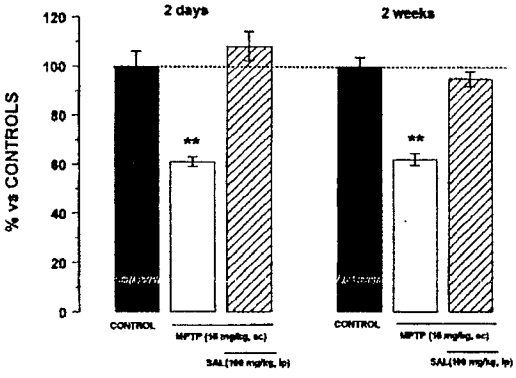

FIG. 3. Protective effects of salicylate (SAL) on depletion of DA in striatum of mice induced by MPTP 2 days or 2 weeks after treatments. The animals received a single administration of SAL (100 mg/kg, i.p.) 1 h before MPTP (15 mg/kg, s.c.) and were killed 2 days or 2 weeks later. Data are mean ± SEM (bars) values (n = 6). Control DA levels were 11,799 ± 704 and 15,452 ± 544 pg/mg of tissue at 2 days and 2 weeks, respectively. $^{**}p < 0.01$ compared with the respective control.

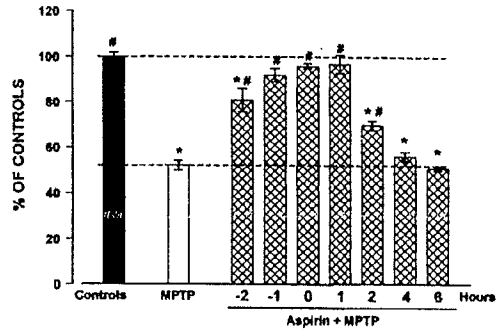

FIG. 5. Time course of protective effects of aspirin on MPTP-induced depletion in DA level in mouse striatum. The animals received a single administration of aspirin (100 mg/kg, i.p.) at different times before or after MPTP (15 mg/kg, s.c.) and were killed 2 days later. Data are mean ± SEM (bars) values (n = 6). Control DA levels were 14,450 ± 879 pg/mg of tissue. $^{*}p < 0.01$ compared with control; $^{\#}p < 0.01$ compared with MPTP group.

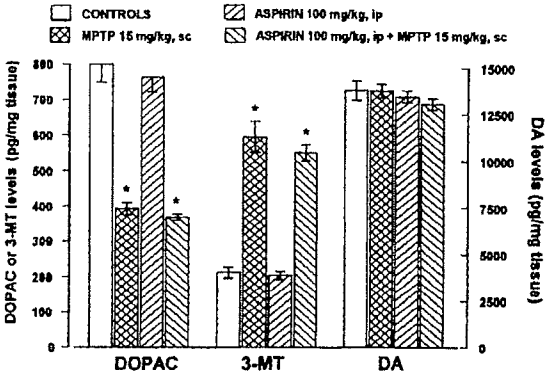

FIG. 6. Acute effects of MPTP (15 mg/kg, s.c.) and aspirin (100 mg/kg, i.p.) alone or combined on striatal DA metabolism. Vehicle or aspirin was injected 1 h before MPTP injection. The effects of MPTP on striatal levels of DA, DOPAC, and 3-MT were measured 30 min after injection of MPTP or 1 h 30 min after injection of aspirin. Data are mean ± SEM (bars) values (n = 8). *$p < 0.01$ compared with control.

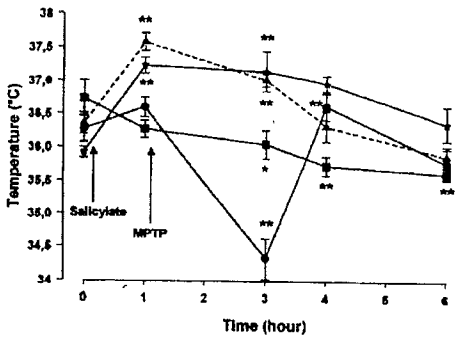

FIG. 7. Time course of effects of MPTP (15 mg/kg, s.c.) and salicylate (100 mg/kg, i.p.) alone or in combination on the core temperature of mice. Salicylate (100 mg/kg, i.p.) or vehicle was administered 1 h before MPTP (15 mg/kg, s.c.): controls (■), MPTP (●), salicylate (▲), and salicylate plus MPTP (★). Data are mean ± SEM (bars) values (n = 6). *$p < 0.05$, **$p < 0.01$ versus respective control (core temperature before salicylate or vehicle administration).

1B. MPTP (15 mg/kg, s.c.) decreased striatal DOPAC and increased striatal 3-MT levels 30 min after injection, without at this early stage reducing striatal DA levels (Fig. 6).

Aspirin per se (100 mg/kg, i.p.) had no effect on DA, 3-MT, or DOPAC levels (Fig. 6) 1 h 30 min after injection, and when administered 1 h before MPTP did not modify the MPTP-induced acute increase in 3-MT or decrease in DOPAC levels.

Effect of MPTP on salicylate and DHBA striatal levels after administration of aspirin

It has already been shown that MPTP increases the cerebral hydroxylation of systemically administered salicylate (Chiueh et al., 1992, 1993). When aspirin (100 mg/kg, i.p.) was administered to mice, high levels of salicylate and its hydroxylated metabolites, 2,3- and 2,5-DHBA, were detected in striatal samples 1 h 30 min later (Table 1). When MPTP (15 mg/kg, s.c.) was given 1 h following aspirin administration, 30 min before the animals were killed, a marked increase in the levels of the hydroxylated metabolites, 2,3-DHBA ($p < 0.01$) and 2,5-DHBA ($p < 0.05$), was observed (Table 1). The ratios of 2,3-DHBA/salicylate and 2,5-DHBA/salicylate were increased by 158 and 66%, respectively, by MPTP administration.

Systemic administration of the hydroxylated metabolites of salicylate (2,3- and 2,5-DHBA (100 mg/kg, i.p.) was without effect on the DA depletion induced by MPTP (data not shown).

Effects of MPTP, salicylate, aspirin, and Aspegic on body temperature of mice

As illustrated in Fig. 7, MPTP (15 mg/kg, s.c.) induced a transient decrease in core temperature with a maximal effect of −2°C, 2 h after administration. This decrease was blunted by salicylate administered 1 h before MPTP. Salicylate alone or in combination with MPTP produced a small but significant increase in core temperature from 1 h up to 6 h after administration.

Lack of effect of salicylate, aspirin, and Aspegic on synaptosomal DA uptake and on MAO-A and MAO-B activities

Salicylate or aspirin (100 μM) had no significant effect on striatal [^{3}H]DA uptake in rat striatal synaptosomes or on MAO-A or MAO-B activity in rat brain homogenates. DA uptake values were 64.0 ± 5, 57.6

TABLE 1. *Effect of MPTP on hydroxyl radical formation as assessed by breakdown of salicylate to 2,3- and 2,5-DHBA in mouse striatum after administration of aspirin*

Treatment	Salicylate (nmol/g)	2,3-DHBA (pmol/g)	2,5-DHBA (pmol/g)	2,3-DHBA/salicylate	2,5-DHBA/salicylate
Aspirin	43 ± 8	12.6 ± 5.8	148 ± 32	0.24 ± 0.07	3.56 ± 0.42
Aspirin + MPTP	57 ± 10[a]	40.1 ± 11[b]	355 ± 79[c]	0.62 ± 1.1[b]	5.91 ± 0.46[c]

The animals received a single administration of aspirin (100 mg/kg, i.p.) 1 h before MPTP (15 mg/kg, s.c.) and were killed 30 min later. Data are mean ± SEM values (n = 8).

[a] Not significant, [b] $p < 0.05$, [c] $p < 0.01$ compared with aspirin group.

TABLE 2. *Effects of diclofenac, indomethacin, ibuprofen, paracetamol, and dexamethasone on reduction in striatal DA levels in mice produced by MPTP*

	Diclofenac (100 mg/kg, i.p.)	Indomethacin (100 mg/kg, i.p.)	Ibuprofen (20 mg/kg, i.p.)	Paracetamol (100 mg/kg, i.p.)	Dexamethasone		
					3 mg/kg, s.c.	10 mg/kg, s.c.	30 mg/kg, s.c.
Control	13,763 ± 832	13,763 ± 832	11,799 ± 704	13,805 ± 219	14,480 ± 359	14,480 ± 359	14,480 ± 359
MPTP (15 mg/kg, s.c.)	5,640 ± 305[a]	5,640 ± 305[a]	7,257 ± 249[a]	7,545 ± 137[a]	7,570 ± 310[a]	7,570 ± 310[a]	7,570 ± 310[a]
MPTP + drug	4,957 ± 907[a]	7,522 ± 268[a]	6,954 ± 113[a]	8,047 ± 152[a]	8,600 ± 395[a]	8,188 ± 352[a]	8,657 ± 238[a]

Drugs were administered 1 h before MPTP, and the mice were killed 2 days later. DA levels are mean ± SEM values ($n = 8$ striata), in pmol/mg of tissue.

[a] $p < 0.001$ versus control.

± 6, and 62.7 ± 4 pmol/min/mg of protein in the control, aspirin (100 μM), and salicylate (100 μM) groups, respectively. MAO-A activity values were 0.130 ± 0.06, 0.123 ± 0.05, and 0.126 ± 0.05 nmol/min/mg of tissue in control, aspirin (100 μM), and salicylate (100 μM) groups, respectively. The corresponding values for MAO-B activity were 0.108 ± 0.06, 0.108 ± 0.05, and 0.105 ± 0.06 nmol/min/mg of tissue.

Effect of cyclooxygenase inhibitors and dexamethasone on MPTP-induced DA depletion

Paracetamol (100 mg/kg, i.p.), ibuprofen (20 mg/kg, i.p.), indomethacin (100 mg/kg, i.p.), diclofenac (100 mg/kg, i.p.), or dexamethasone (3, 10, and 30 mg/kg, s.c.) was administered 1 h before MPTP (15 mg/kg, s.c.). None of these drugs decreased the neurotoxic effect of MPTP (Table 2).

DISCUSSION

Salicylate, aspirin, and its soluble lysine salt, Aspegic, are able to protect totally against the neurotoxic effects of MPTP in mice. Protection by salicylate was maintained for at least 2 weeks after MPTP administration and is not a transient effect. The neuroprotective effects of aspirin were fully maintained when aspirin was administered 1 h after MPTP treatment, and significant (but reduced) protection was still observed with an administration delay of 2 but not 4 h. The process with which aspirin interacts is thus manifest within the first 4 h following MPTP administration. During this time hydroxyl radical production is evident (Chiueh et al., 1992, 1994; Obata and Chiueh, 1992). The maximal decrease in mitochondrial complex 1 activity is observed ~1 h following MPTP administration in mice (Sriram et al., 1997). None of these drugs affected DA uptake or MAO-B activity, and their neuroprotective effects are therefore related to interference with a neurotoxic process and not to blockade of MPTP metabolism or MPP^+ uptake into dopaminergic terminals. Within the first hours of administration, MPTP results in an acute increase in striatal 3-MT levels coupled with a reduction in striatal DOPAC levels, likely related to its immediate effects on DA uptake and metabolism, as shown by Pileblad et al. (1985). These acute effects of MPTP were totally unaffected by salicylate, suggesting that salicylate does not interfere with the cerebral entry or metabolism of MPTP itself. Salicylate was less protective against a higher dose of MPTP, suggesting some type of competitive effect between salicylate and the neurotoxic effector. The effects of salicylate cannot be explained in terms of hypothermia, and indeed salicylate appeared to produce slight hyperermic effects per se. The hypothermic effects of MPTP are likely related to its acute neurotoxic effects, and their antagonism by salicylate is a symptomatic manifestation of its neuroprotective action. It seems unlikely that cyclooxygenase inhibition explains the neuroprotective effects of aspirin. Salicylate, which was also effective in this model, is only a weak inhibitor of cyclooxygenase (Mitchell et al., 1994), and other reference cyclooxygenase inhibitors [acetaminophen (paracetamol), diclofenac, ibuprofen, and indomethacin (Mitchell et al., 1994)] were ineffective against the neurotoxic effects of MPTP.

Aspirin and salicylate (at millimolar concentrations) have both been reported to inhibit the activation of the transcription factor NF-$\kappa\beta$ in various in vitro models (Kopp and Ghosh, 1994; Grilli et al., 1996). Diverse noxious cellular stimuli free NF-$\kappa\beta$ from an endogenous inhibitor, allowing translocation of free NF-$\kappa\beta$ from the cytoplasm to the nucleus. NF-$\kappa\beta$ then binds to DNA and activates several genes involved in inflammatory and immune responses. Some of these gene products, for example, tumor necrosis factor-α, may exert cytotoxic effects by switching on apoptotic self-destruct programs (Wright et al., 1992; Vaux and Strasser, 1996). Apoptosis has been reported to be a feature of MPP^+-related cytotoxicity (Mochizuki et al., 1994). The effects of MPTP or MPP^+ on NF-$\kappa\beta$ activation do not appear to have been studied, although increased translocation of NF-$\kappa\beta$ has been observed in Parkinson's disease brain (Hunot et al., 1997). Both aspirin and salicylate block the glutamate-induced activation of NF-$\kappa\beta$ in rat cerebellar granule cells and furthermore block the neurotoxicity of glutamate on

cerebellar granule cells and in hippocampal slices (Grilli et al., 1996). As indomethacin does not block NF-$\kappa\beta$ activation or glutamate toxicity in vitro (Grilli et al., 1996) and did not protect against the neurotoxic effects of MPTP, it is possible that NF-$\kappa\beta$ inhibition is somehow involved in the MPTP neurotoxic cascade and in the protective effects of aspirin. However, dexamethasone, which is a much more potent (nanomolar) inhibitor of NF-$\kappa\beta$ activation in similar in vitro models (Auphan et al., 1995), was totally ineffective against MPTP toxicity.

Nitric oxide synthase inhibitors have been reported to block the neurotoxic effects of MPTP (Schulz et al., 1995*b*; Przedborski et al., 1996). Aspirin does inhibit nitric oxide synthase at high concentrations (IC_{50} of ~1 m*M*), but salicylate is without effect (Amin et al., 1995), and it seems unlikely that nitric oxide synthase inhibition explains these neuroprotective effects. It has also been suggested that the protective effects of certain nitric oxide synthase inhibitors may in fact be related to their additional ability to inhibit MAO-B (Di Monte et al., 1997).

Salicylate is an effective hydroxyl radical trapping agent, and indeed an increase in levels of the hydroxylated metabolites of salicylate following MPTP coadministration was initially used to show that MPTP generates hydroxyl radicals in vivo (Chiuch et al., 1992, 1993; Obata and Chiueh, 1992). Aspirin also effectively traps hydroxyl radicals (Halliwell et al., 1987) and is rapidly metabolized to salicylate following systemic administration (Gaspari et al., 1989). Following the administration of aspirin, our results show that salicylate and its hydroxylated metabolites can be found in the mouse brain. The production of these hydroxylated metabolites is increased by MPTP, confirming that MPTP generates hydroxyl radicals that react with salicylate derived from aspirin. Hydroxyl radical scavenging activity is thus one possible explanation for the neuroprotective effects of aspirin and salicylate. Other free radical scavengers have been reported to be effective in the MPTP model, although it has to be said that their neuroprotective effects have not been as impressive (Perry et al., 1985; Akaneya et al., 1995; Schulz et al., 1995*a*). It should also be mentioned that a very potent hydroxyl radical scavenger, dimethyl sulfoxide (Gaspari et al., 1989), does not protect against the neurotoxic effects of MPP^+ in vitro (Akaneya et al., 1995).

At this stage, and without further experiments designed specifically to address the question, it is probably wise to reserve judgment on the mechanism of neuroprotective action of aspirin and salicylate, although one can at least rule out cyclooxygenase inhibition. It is nevertheless surprising and exciting that these two drugs are able to prevent completely neurotoxic effects in this animal model of Parkinson's disease—an observation that merits close clinical attention.

Aspirin is known to produce toxic effects at high doses in humans (for example, acidosis and gastrointestinal bleeding). In these animal experiments, the results suggest some form of competition between aspirin and the toxic effector produced by MPTP, i.e., the higher the dose of MPTP, the more aspirin is needed to combat its effects. In the animal studies, dopaminergic toxicity is produced by a bolus injection of a single high dose of MPTP, and very high doses of aspirin or salicylate are necessary to combat its effects. In the clinical situation one would hope that the long time course of the degenerative process in Parkinson's disease reflects chronic toxicity of lower levels of any putative MPTP-like neurotoxin. If this were the case, it is possible that relatively low repeated doses of aspirin or salicylate might be able to slow disease progression.

In clinical trials, aspirin has been shown to be of clear benefit in the prevention of Stroke (Antiplatelet Trialist's Collaboration, 1994), a factor generally attributed to its beneficial effects on platelet aggregation. The accumulating reported neuroprotective effects of aspirin perhaps hint at a more direct effect on neuronal resistance. It has also been noted that there is an inverse correlation between antiinflammatory treatment (including the use of aspirin) and Alzheimer's disease (Breitner et al., 1994). Both aspirin and salicylate block glutamate-induced neurotoxicity in vitro (Grilli et al., 1996) and MPTP-induced neurotoxicity in vivo, via mechanisms that are clearly unrelated to the classical primary effect of aspirin, cyclooxygenase inhibition. There appears to be a hidden aspect of aspirin and salicylate pharmacology whose further characterization may lead to the clearer definition of neurotoxic processes and to the development of novel treatments in various central degenerative diseases.

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